HIF-2a regulates in vitro neovascularization during hypoxia

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Tissue engineering or regenerative medicine refers to the culture of cells on a biodegradable scaffold *ex vivo* and subsequent implantation to repair tissues that cannot heal through tissue repair. The onset of tissue engineering promised the replacement of large or complex damaged organs and regeneration of tissues. Unfortunately, oxygen diffusion is limited in a cellular tissue-engineered scaffold, resulting in reduced (hypoxia) or lack (anoxia) of oxygen within the deeper regions of the scaffold. Moreover, the severe and cell damaging hypoxia in the tissue-engineered graft often gives resistance to induce vascularization. Consequently, the hypoxic core expands due to resistance to vascularization, eventually leading to a necrotic area. Therefore, there is a need of overcoming either the excess of inhibitory factors or the lack of stimulatory factors that result in reduced induction of angiogenesis in severely hypoxic tissues.

Angiogenesis is important for growth, development and proper wound healing, but is also associated with several pathological conditions. Metabolic and inflammatory disorders are often accompanied by inadequate blood supply or enhanced metabolic demand, leading to hypoxia in the tissue. Not surprisingly, hypoxia is considered to be one of the most potent initiators of angiogenesis *in vitro* and *in vivo*. Although a short exposure to hypoxia stimulates angiogenesis, prolonged hypoxia in poorly perfused or healing tissues is often accompanied by a resistance to induce neovascularization. It is suggested that the balance between stimulating capillary sprouting and stabilizing the endothelial sprout is important for proper angiogenesis. This is regulated through the Hypoxia Inducible Factors (HIF) -1α and -2α. Although HIF-1α and HIF-2α regulate many similar genes in response to hypoxia, their role in angiogenesis is different; HIF-1α stimulates angiogenesis-related processes such as endothelial sprouting and proliferation, whereas HIF-2α stimulates vessel remodeling into mature and functional vessels or strengthening of the endothelial barrier.

The main aim of this thesis is to evaluate the effect of hypoxia and HIF-2α on the regulation of both the initiation and the stabilization of the endothelial sprout during angiogenesis. Objectives of this study are: 1) to clarify the effect of prolonged hypoxia on HIF-2α expression and endothelial sprouting (discussed in **Chapter 3**). 2) To unravel which HIF-2α-downstream genes and pathways regulate the initiation of sprouting (discussed in **Chapter 4**). 3) To establish through which mechanisms hypoxia and HIF-2α regulate endothelial barrier function (discussed in **Chapter 5**). 4) To explore the involvement of mitochondria in endothelial sprouting (discussed in **Chapter 6**).
This thesis starts with a short introduction on angiogenesis, hypoxia and the HIFs (**Chapter 1**), followed by the aim and objectives of the study. **Chapter 2** discusses the role of HIF-1α and HIF-2α in the regulation of angiogenesis during embryogenesis and wound healing and possible application in tissue engineering.

During hypoxia, HIF-1α and HIF-2α are stabilized and regulate the expression of many genes involved in amongst others angiogenesis. However, during some pathological conditions, prolonged hypoxia occurs and is accompanied by reduced angiogenesis. In **Chapter 3** we investigated the effect of prolonged hypoxia on the proliferation and sprouting ability of human microvascular endothelial cells and the involvement of the HIFs. Prolonged hypoxic precultured endothelial cells lose their ability to form sprouts. Two independent mechanisms contribute; silencing of HIF-2α with si-RNA partially restores the inhibition of endothelial sprouting during prolonged hypoxia pointing to a HIF-2α-dependent mechanism. In addition, reduction of uPA contributes to reduced endothelial tube formation in a fibrin matrix during prolonged hypoxia. We used recombinant uPA to increase the tube formation, therefore a direct comparison of the quantification of this effect with that by endogenous uPA should be made with caution.

In **Chapter 4**, we identified novel HIF-2α-target genes that may regulate endothelial sprouting during prolonged hypoxia. Many genes are significantly differentially regulated in hypoxia or upon HIF-2α silencing, identified by using genome-wide RNA-sequencing. Only 51 genes are regulated by both mechanisms in opposite directions. Evaluation of these 51 genes reveals that four genes directly affect endothelial sprouting, i.e. ARRDC3, MME, PPARG, and RALGPS2.

Endothelial cells form a tight barrier to prevent vascular leakage. The effect of hypoxia on the vascular leakage is controversial. **Chapter 5** investigates how hypoxia and the hypoxia-mimetic dimethyloxalylglycine (DMOG) affect adherens junction integrity and barrier function of human endothelial monolayers and which mechanisms are involved. We show that hypoxia and DMOG both reduce vascular leakage, which was dependent on HIF-2α but not HIF-1α. HIF-2α stabilizes adherens junctions through VE-cadherin.

Mitochondria have been reported to contribute to the induction of HIF-1α and HIF-2α during hypoxia, but the involvement of mitochondria during endothelial sprouting is not clear. Therefore, we investigate the effect of prolonged hypoxia on mitochondria number and respiration and the involvement of mitochondria in endothelial sprouting in **Chapter 6**. Mitochondrial enzyme expression is reduced during prolonged hypoxia, which results in a lower mitochondrial density, a lower maximal capacity of the electron transport system, and reduced production of oxygen radicals. However, according to our data, it is unlikely that reduced mitochondrial ATP production determines the markedly reduced endothelial sprouting by endothelial cells exposed to prolonged hypoxia.

Finally, **Chapter 7** summarizes and discusses the results of this thesis in the context of previous and recent developments in the field of angiogenesis and hypoxia. Furthermore, a brief overview of the conclusions and future directions are provided.

This thesis shows that hypoxia and HIF-2α influence multiple steps of the angiogenesis cascade. HIF-2α plays an important role in endothelial sprouting as well as stimulating vessel remodeling and strengthening of the endothelial barrier. As these actions are probably regulated by different HIF-2α-downstream targets, it is important to investigate which targets influence which pathways. Therefore, manipulating specific downstream targets of HIF-2α provides a new to be further evaluated perspective for restoring reduced neovascularization for example during tissue engineering.